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Portable optical spectroscopic assay for non-destructive measurement of key metabolic parameters on *in vitro* cancer cells and organotypic fresh tumor slices

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Abstract: To enable non-destructive metabolic characterizations on *in vitro* cancer cells and organotypic tumor models for therapeutic studies in an easy-to-access way, we report a highly portable optical spectroscopic assay for simultaneous measurement of glucose uptake and mitochondrial function on various cancer models with high sensitivity. Well-established breast cancer cell lines (MCF-7 and MDA-MB-231) were used to validate the optical spectroscopic assay for metabolic characterizations, while fresh tumor samples harvested from both animals and human cancer patients were used to test the feasibility of our optical metabolic assay for non-destructive measurement of key metabolic parameters on organotypic tumor slices. Our optical metabolic assay captured that MCF-7 cells had higher mitochondrial metabolism, but lower glucose uptake compared to the MDA-MB-231 cells, which is consistent with our microscopy imaging and flow cytometry data, as well as the published Seahorse Assay data. Moreover, we demonstrated that our optical assay could non-destructively measure both glucose uptake and mitochondrial metabolism on the same cancer cell samples at one time, which remains challenging by existing metabolic tools. Our pilot tests on thin fresh tumor slices showed that our optical assay captured increased metabolic activities in tumors compared to normal tissues. Our non-destructive optical metabolic assay provides a cost-effective way for future longitudinal therapeutic studies using patient-derived organotypic fresh tumor slices through the lens of tumor energetics, which will significantly advance translational cancer research.

1. Introduction

Tumor metabolism reprogramming has been recognized as one of the cancer hallmarks [1]. The metabolic rewiring not only provides an unparalleled advantage to tumor cells to survive, grow, and metastasize under a hypoxic and nutrient-poor environment but also endows these cells with unlimited plasticity to adapt and escape cancer therapies [2]. It is well known that many tumors employ high rates of glycolysis during normoxia (Warburg effect) [3]. Beyond the extensively studied "Warburg effect" [3], mitochondria have also gained recognition for their distinct contribution to tumor oxidative metabolism [4]. New studies show that some aggressive tumors can "adapt" between glycolysis and oxidative phosphorylation (OXPHOS) to survive under hostile conditions and therapeutic stress [2,5]. Therefore, both glycolysis and mitochondrial function of tumors could impact therapeutic outcomes and can be used as effective biomarkers in a wide range of cancer applications.

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To maximize cancer patient' survival, *in vitro* cancer cells [6] and newly developed patient-derived organotypic tumor models [7,8] are widely used to study the role of tumor metabolism in tumor growth and survival under therapeutic stress. As patient-derived organotypic tumor models retain the original behavior and responsiveness of primary tumors [7,8], they have been explored as the state-of-art clinically relevant cancer models for translational studies [9–11] albeit they are expensive and difficult to harvest and maintain. In typical therapeutic studies, patient-derived organoids or tumor slices are cultured and kept alive for a few weeks during which the treatments will be directly applied on the organoids or tumor slices for therapeutic response pre-evaluations [12]. To provide direct evaluations of the therapeutic response of organotypic tumor samples before and after treatments and minimize the translational cost, it is critical to be able to conduct longitudinal non-destructive studies on the same sample [9]. However, few techniques can provide non-destructive metabolic measurements on organotypic tumor models. The most commonly used metabolic tool for assessing glycolysis and OXPHOS is Seahorse Assay, which measures the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of *in vitro* cells [13,14], while destructive chemical perturbations on cells are needed. The gold standard tool for measuring metabolites is metabolomics, which can quickly screen a large number of molecules and map metabolic networks from *in vitro* cells to *ex vivo* tissues [15]. However, the destructive process, i.e. metabolite extraction using biochemistry procedures, is essential for metabolomics measurements [16]. Therefore, it is impossible to conduct longitudinal measurements on the same tumor sample using either Seahorse Assay or metabolomics due to their destructive nature.

Optical microscopy can enable non-destructive metabolic imaging on various cancer models [17] using either endogenous contrast or appropriate indicators. Autofluorescence of reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) in cells has been explored [18] to report the reduction-oxidation (redox) state of cancer cells [19]. Optical redox ratio techniques have been utilized extensively to report patient-derived organoids drug therapeutics responses [9,20]. Several cutting-edge microscopes including confocal and multiphoton were explored as state-of-art optical metabolic tools for cellular-level imaging of *in vitro* cancer cells or organoids [9,20]. However, these advanced microscopes have limited user access due to their high-cost and low portability. To quantify tumor cell glycolysis and mitochondrial function directly and explicitly at a lower cost, we exploited metabolic probes-based approaches to report glucose uptake and mitochondrial function using visible light-based single photon fluorescence microscopes [21]. Specifically, 2-NBDG was used to report glucose uptake in cancer cells, similar to the clinically available FDG-PET [22,23]. TMRE was utilized to quantify cell mitochondrial membrane potential (MMP), which has been explored to study OXPHOS [21,24]. Bodipy has been also recently explored to report cancer cells' fatty acid uptake [25]. When these metabolic probes were used, a rapid labeling procedure was required but it was completely non-destructive [26]. We have implemented these fluorescence probes with microscopy techniques for metabolic imaging on various cancer models including *in vitro* cancer cells [26] and *in vivo* small animals [21,27]. However, our current metabolic microscopes also have limited user access due to the low portability and moderate instrument cost.

To enable non-destructive metabolic characterizations on organotypic tumor models for therapeutics studies in an easy-to-access way, here we report a portable optical spectroscopic assay for simultaneous measurements of glucose uptake and mitochondrial functions on various cancer models. Specifically, we developed a highly portable fluorescence spectroscopy assay using a custom-designed low-cost dual-color LED illuminator and a compact spectrometer, along with a low-cost fiber probe and optical filters. We then used well-characterized breast cancer cell lines (MCF-7 and MDA-MB-231) to validate the assay with flow cytometry and microscopy for metabolic characterizations. We also utilized fresh tumor samples harvested from animals and human breast cancer patients to test the feasibility of the optical assay for non-destructive measurements of key metabolic parameters on organotypic tumor slices. In our *in vitro* cell

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studies, our optical assay captured that MCF-7 cells had a higher mitochondrial metabolism but a lower glucose uptake compared to the MDA-MB-231 cell, which is consistent with our microscopy imaging and flowcytometry data, as well as the published Seahorse Assay data [28]. Moreover, we demonstrated that our spectroscopic assay could measure both glucose uptake and mitochondrial metabolism on the same cancer cell samples at one time, which remains challenging for other existing metabolic tools. Our pilot tests on fresh tumor slices showed that our optical assay captured increased metabolic activities in tumors compared to normal tissues. Through these studies, we demonstrated a cost-effective portable tool to enable non-destructive metabolic characterizations on *in vitro* cancer cells and organotypic fresh tumor slice models. Our non-destructive optical assay provides a new way to enable longitudinal therapeutic studies on organotypic tumor models through the lens of tumor energetics, which will significantly advance translational cancer research.

2. Materials and methods

2.1. Portable low-cost optical spectroscopy platform and its basic characterization

A portable optical spectroscopy system was built based on a custom designed low-cost high-power dual-color LED illuminator (Luxeon LEDs) and a compact spectrometer (FLAME-T-VIS-NIR, Ocean Optics), along with a low-cost fiber probe and optical filters using the layout in Fig. 1(A) to maximize its portability with minimal cost.

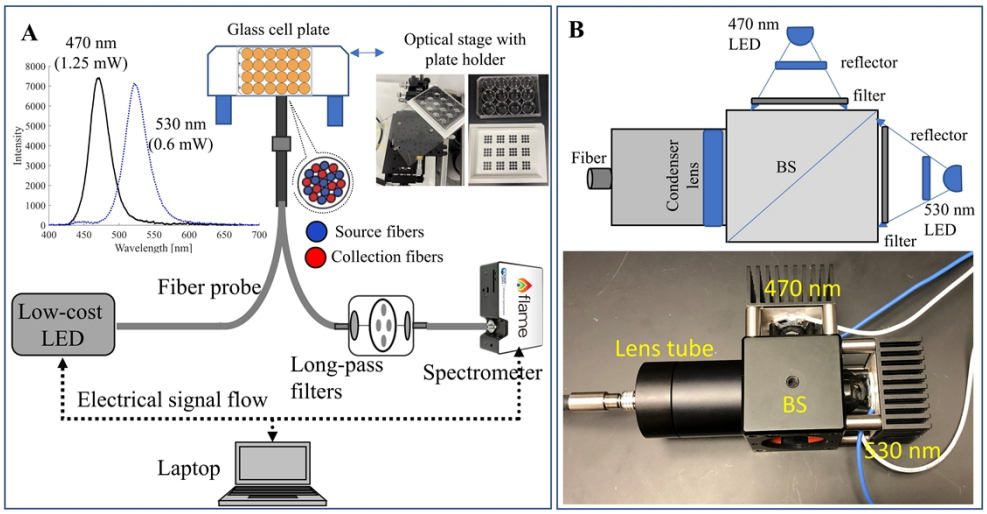

Fig. 1. **A** Schematic of the portable optical spectroscopic assay along with the custom-designed cell culture plate holder. **B.** Schematic and the actual photo of the low-cost dual-color LED illuminator.

A commercially available fiber probe (BF19Y2HS02, Thorlabs) with 10 illumination fibers and 9 collection fibers was used for light delivery and collection. The diameter of each fiber is 200 μm, and the illumination and collection fibers were evenly and adjacently bundled in the common end with an average optical sampling area of ~1 mm in diameter. Two LEDs, one 530 nm LED (Luxeon, 07040-PM000-L) and one 470 nm LED (Luxeon, J035-L1C1BLU), were used to build the illuminator as illustrated in Fig. 1(B). A beam splitter (50/50) combines the two colors of light to the main fiber probe for sample illumination. A reflector and a condenser lens were used in front of LEDs to focus as much power as possible on the illumination fibers and minimize any potential illumination light leakage. Optical bandpass or short pass filters were used next to the blue LED (450 nm ±30 nm bandpass) and the green LED (550 nm cutoff

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short pass) to narrow the excitation light bands. The heating sinks were used next to each LED to ensure they will deliver stable power. A switch controller circuit was used to turn on/off the two LEDs sequentially as needed. The blue light was used for 2-NBDG excitation (excitation peaks: 480 nm), and the green light was used for TMRE excitation (excitation peak: 540 nm). In the collection end, two long-pass filters (515 nm and 575 nm) were used to block any potential excitation light during fluorescence measurements. To implement the spectroscopy platform with *in vitro* cancer cell models or organotypic tumor models, a custom-designed cell culture plate holder (Fig. 1(A)) was printed using a 3D printer and then mounted to an optical stage for optical measurements. To test the illuminator power stability, the LEDs were turned on continuously for 2 hours and the corresponding power output was recorded by an optical power meter (PM120D, Thorlabs). To characterize the system's sensitivity for quantifying the metabolic probes, the fluorescent probes were dissolved in Phosphate Buffered Saline (PBS) 1x (Fisher Scientific) at biological concentrations. Specifically, the 2-NBDG was varied from 0 to 400 nM by every 100 nM, and the TMRE was varied from 0 to 40 nM by every 10 nM based on our former *in vitro* imaging studies [26]. Liquid phantoms were added to the cell culture plate and then the plate was fixed in the plate holder for fluorescence measurements with an integration time of 2 seconds. The key optical components used to build the assay have been summarized in Table 1. The total cost for these components used in our system was around $3500. The total weight of the system is less than 2 lbs which makes the system highly portable.

Table 1. Key optical components used in the portable optical assay system

Illumination	Vendor and item ID	Collection	Vendor and item ID
Blue LED	J035-L1C1BLU, Luxeon	Fiber probe	BF19Y2HS02, Thorlabs
Green LED	07040-PM000-L, Luxeon	Long pass filter (515 nm)	FGL515, Thorlabs
Bandpass filter	#84-095, Go Edmund Optics	Long pass filter (575 nm)	#84-745, Go Edmund Optics
Short pass filter	FELH0550, Thorlabs	Filter wheel	CFW6, Thorlabs
Reflector	10193, LEDsupply	Spectrometer	FLAME-T-VIS-NIR, Ocean Optics
Heating sink	HS13137, LEDsupply	Collection fiber	BFL200LS02, Thorlabs
Beam splitter	CCM1-BS013, Thorlabs	Condenser lens	ACL2520U, Thorlabs

2.2. Cell culture and metabolic measurements

Breast cancer cell lines MDA-MB-231 and MCF-7 (ATCC, Manassas, VA) were used in our optical assay validation study since their metabolic profiles have been well-characterized by others [28,19]. Both cell lines were cultured in DMEM/F12 (Gibco) media supplemented with 10% FBS (Gibco) and 1X penicillin-streptomycin (Gibco) at 37°C and 5% CO_2 incubator. Cell medium was replaced every two days with fresh media. The cells were cultured in 12-well culture plates, and they were used for metabolic measurements when the cell confluent reached ~80%. The 2-NBDG and TMRE [21] were used in the metabolic measurements using the optical assay, microscopy, and flow cytometry. The 2-NBDG (Biogems) is an optical analog glucose that enters the cell via glucose transporters [29,26], which measures glucose uptake analogous to widely accepted PET imaging [30]. The TMRE (Biogems) is a cationic dye that accumulates in the mitochondrial inner membrane as a function of MMP [31], which has been utilized extensively to study mitochondrial capability [21,23]. Before any metabolic characterization, the regular media was removed and the cells were washed with PBS (Gibco) twice and then incubated at 37∘C and 5% CO_2 for up to 60 minutes with either 200 mmol/L 2-NBDG or 50 nmol/L TMRE dissolved in glucose-free media [26]. In microscope imaging and flowcytometry experiments, cells were only stained with one of the two probes to serve as controls. However, in the optical assay measurements, cells were stained with either one probe (TMRE only or 2-NBDG only) or

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two probes (TMRE first and then 2-NBDG with a 30 minute delay [21]) to explore the feasibility for simultaneous measurements of glucose uptake and mitochondrial membrane potential on the same cell populations using spectroscopy techniques.

All spectroscopy measurements were conducted using the optical platform shown in Fig. 1. The cell culture plate with breast cancer cells was fixed in the plate holder for spectral measurements with an integration time of 2 seconds. The cell confluence for each scanned region (pre-selected by the plate holder) was further confirmed by a microscope. All optical images were collected using a ZOE Fluorescent Cell Imager (Bio-Rad). The green channel was used for 2-NBDG uptake imaging, the red channel was used for TMRE uptake imaging. The gain level, contrast level, and LED intensity of the microscope were optimized and then fixed for either 2-NBDG or TMRE imaging to ensure that images with sufficient optical signals could be obtained and compared. In flow cytometry experiments, the cells were detached by 0.25% Trypsin (Gibco) after probe labeling and then suspended at 1500 rpm for 5 minutes by a centrifuge (Fisher Scientific). The cells were washed three times with PBS and then analyzed using a flow cytometer (BD FACSymphony A3 Cell Analyzer) with optimized configurations.

2.3. *Ex vivo fresh tumor slices and optical spectroscopy measurements*

Preclinical animal tumors were used as a cost-effective model to optimize the optical assay protocol for metabolic measurements on tumor slices. The xenograft tumors were generated subcutaneously by injecting SCC-61 cells suspended in half PBS and half Matrigel matrix (Corning) on the flank of 6-week-old nude mice. Once the tumor diameter reached 5 mm, the mice were fasted for 6 hours and received a tail-vein injection of TMRE(100 µL of 100 µM), or 2-NBDG (100 µL of 6 mM), or both probes (TMRE first and then 2-NBDG with a 15-minute delay [23]). Around 60 minutes (single probe group) or 75 minutes (two probes group) post tail vein injection, the tumor tissue and normal tissues were harvested, sliced (~2 mm), and then kept in 12-well glass bottom culture plates (Cellvis) in cold PBS. Optical spectroscopy measurements were immediately conducted on the fresh tumor samples using the proposed optical platform to optimize the optical configurations such as the integration time. All animal studies were conducted according to a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

2.4. *Patient-derived organotypic tissue cultures (OTC) and optical metabolic spectroscopy pilot test*

To demonstrate the feasibility of the optical assay for non-destructive measurements of key metabolic parameters on clinical organotypic tumor tissue cultures, a small pilot study was conducted using human patient breast tumor samples provided by the Biospecimen Procurement & Translational Pathology Shared Resource Facility of the Markey Cancer Center under a protocol approved by the University of Kentucky Institutional Review Board (IRB). Patient-derived breast tumor tissues were harvested, thinly sliced, and cultured following a protocol modified from two previous reports [32,33] with some improvements that will be reported in a future paper. Briefly, fresh patient breast tumors and matched normal breast tissue samples were transported in DMEM/F12 (Gibco) from the hospital to the research laboratory within 2 hours post-surgery. The tissue chunk was embedded in 3% low-melting agarose (Sigma-Aldrich) in a Krumdieck holder after discarding excess fat tissue using surgical tools. After gelling, tissue was cut at 750 µm thickness (Krumdieck Tissue slicer) in cold PBS. The tissue slices were rinsed with PBS, excess liquid removed, and weighed (Sartorius). Then tissue slices were spread in a 12 mm Snapwell Insert with 0.4 µm pore membrane (Corning) and covered with 0.5 mL Matrigel (Corning): cultured medium (1:1) mixture in each insert. The Snapwell cell culture inserts were incubated at 37∘C, 5% CO_2 for 30 minutes until the Matrigel matrix was solidified, and then 2.2 mL pre-warmed medium (modified from [32]) was added to each well. Medium change was

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performed regularly every 2-3 days based on the extent of medium acidification. On day 14 post OTC culturing, the slices were used for metabolic assay measurements. The metabolic probe labeling procedure used for tumor slices was the same as that used in cell studies as described above. After labeling, the patient samples were washed with PBS and kept in a 12-well glass bottle plate (Cellvis) for spectroscopy assay measurements. The optical configurations optimized in the preclinical studies were directly used for patient sample characterization.

2.5. Data analysis

Flow cytometry data were analyzed using FlowJo software provided by BD Biosciences. Optical spectroscopy data and microscopy images were processed using MATLAB (MathWorks). The whole spectra of 2-NBDG (500 nm - 700 nm) and TMRE (560 nm -700 nm) were plotted to compare the spectra shape changes among different groups, while the mean of the peak-band (emission peak ± 5 nm) fluorescence intensity of 2-NBDG (emission peak 545 nm) and TMRE (emission peak 585 nm) spectra were used to represent the 2-NBDG and TMRE uptake. The mean intensities for each group were summarized and compared using the student's t-test. The microscope imaging data was pre-processed to count the optical signals on the cells only by removing the background noise, which is defined by the average intensity from the unlabeled cells and culture medium. All pixel values less than this noise threshold value were set to zero. The mean intensities were then created from all nonzero pixels from each image for each group, then the mean intensities for each group were summarized and compared using the student's t-test. Two-sided p-values <0.05 were considered significant. MATLAB Statistics Toolbox was used for all tests.

3. Results

3.1. Power test and phantom studies to characterize the optical system

Figure 2(A) shows the illuminator test result which reports the system power stability for fluorescence excitation. The measured power values were 1.25 mW and 0.62 mW for the blue excitation light (470 nm LED along with 450 nm ±30 nm bandpass filter) and green excitation light (530 nm LED along with a 550 nm short pass filter), respectively. Figure 2(A) shows that the power output was stable for the two colors of excitation lights during the entire 2-hour monitoring period. The power tests were also performed several times on different days (with a shorter monitoring period), and the LEDs always provided the same power outputs which further demonstrated the high stability of the custom-designed low-cost LED illuminator. Figure 2(B-C) shows the fluorescence spectra measured on liquid phantoms that reflect the sensitivity of the optical assay for quantifying the metabolic probes at biologically low concentrations. The 2-NBDG and TMRE spectra shown in Fig. 2(B-C) suggest that the long-pass filters worked well to ensure the capture of the whole spectra of fluorescence from the two metabolic probes with emission peaks at 545 nm and 585 nm, respectively. A linear correlation between the peak fluorescence intensity and fluorophore concentration was observed for each fluorophore as expected ($R^2 = 1.0$ and $p < 0.0001$ for 2-NBDG, $R^2 = 0.999$, and $p < 0.0001$ for TMRE). The phantom studies also demonstrated the measurable changes in 2-NBDG and TMRE intensity at concentration increments as low as 100 nM for 2-NBDG and 10 nM for TMRE.

3.2. Optical assay and flow cytometry capture similar metabolic changes in different breast cell lines

Figure 3 shows the comparison of a flow cytometry and our optical metabolic assay for glucose uptake (2-NBDG uptake) and MMP (TMRE uptake) characterizations on MCF-7 and MDA-MB-231 cell lines. The flow cytometry results show that MCF-7 cells had marginally decreased 2-NBDG uptake (not significant, Fig. 3(A1)) but significantly increased TMRE uptake ($p < 0.05$.

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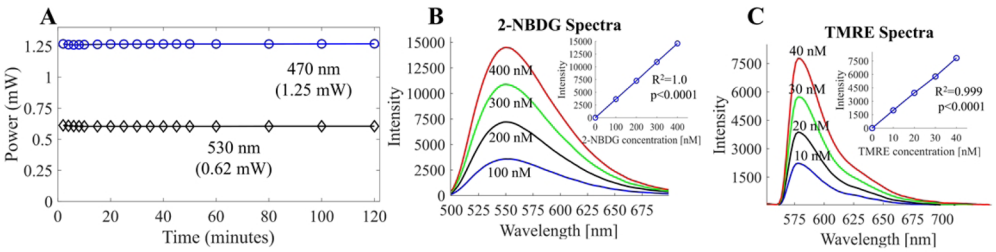

Fig. 2. **(A)** Optical power values of the low-cost LEDs. **(B)** 2-NBDG spectra measured on PBS liquid phantoms. **(C)** TMRE spectra measured on PBS liquid phantoms. The integration time was set to be 2 seconds for both 2-NBDG and TMRE spectra measurements.

Figure 3(A2)) compared to MDA-MB-231 cells. The optical assay measured fluorescence data shows that MCF-7 had significantly decreased 2-NBDG uptake ($p < 0.0001$. Figure 3(B1)) and significantly increased TMRE uptake ($p < 0.00001$. Figure 3(B2)) compared to MDA-MB-231 cells. The statistical analysis on the 2-NBDG and TMRE uptake intensities at their emission peaks (after background subtractions) for both breast cell lines demonstrated the significant differences ($p < 0.0001$) as shown in Fig. 3(C). Figure 3(B-C) show that our optical metabolic assay captured the consistent metabolic changes between MCF-7 and MDA-MB-231 compared to the flow cytometry, but with a high sensitivity as evidenced by the high statistical significance ($p < 0.0001$) for both 2-NBDG and TMRE uptake.

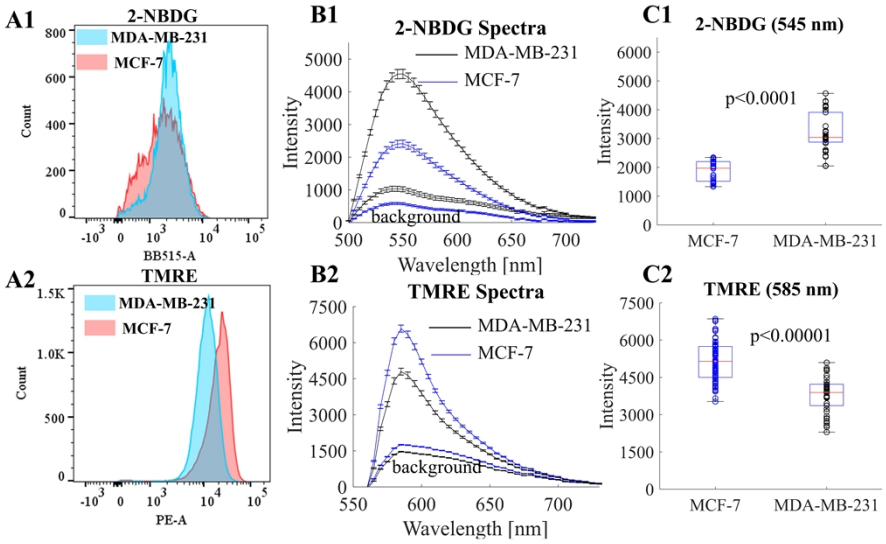

Fig. 3. **(A)** 2-NBDG and TMRE uptake in MCF-7 and MDA-MB-231 cell lines characterized by a flow cytometry. **(B)** Optical assay measured spectra of 2-NBDG and TMRE in MCF-7 and MDA-MB-231 cell lines. **(C)** Statistical analysis of the 2-NBDG and TMRE uptake intensities at their emission peaks after background subtractions for MCF-7 and MDA-MB-231. The number of optical measurements for each set of 2-NBDG and TMRE spectral data was around 40 (around 10 regions in each well for four different wells). The cell confluence for the optical assay measurements was maintained to be 80% for all measured cell wells.

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3.3. Optical assay can simultaneously measure the key metabolic parameters in the same cell population

Figure 4 shows the comparison of a benchtop microscope (ZOE Fluorescent Cell Imager) and our optical metabolic assay for glucose uptake and MMP characterizations on the two breast cancer cell lines. We also explored the feasibility of simultaneous measurements of the two key metabolic parameters on the same tumor cell populations using the optical spectral assay, which remains challenging for most existing benchtop microscopes that have limited spectral capability. Figure 4(A) shows microscopic imaging of 2-NBDG uptake and TMRE uptake on MCF-7 and MDA-MB-231 cell lines. The imaging data showed that MCF-7 cells had significantly lower 2-NBDG uptake, but significantly higher TMRE uptake compared to MDA-MB-231 cells, which is consistent with the findings shown in Fig. 3, as well as published Seahorse Assay data [28]. Figure 4(B-C) shows the comparison of fluorescence spectra between the single probe group (2-NBDG labeling only or TMRE labeling only) and the two-probes group (TMRE labeling first and then 2-NBDG labeling with a 30-minutes delay) for both 2-NBDG and TMRE measurements. Figure 4(B-C) shows that both the spectra shape and intensities were almost identical between the single probe group and the two-probes group for both MCF-7 and MDA-MB-231 cell lines. The statistical analysis on the 2-NBDG and TMRE uptake intensities for both breast cell lines further demonstrated the two-probes approach was statistically identical to the single-probe approach. Figure 4 showed that our optical metabolic assay has similar sensitivity compared to a benchtop fluorescence microscope for glucose uptake and membrane potential characterizations but with the spectral capability to enable simultaneous measurements of the two key metabolic parameters together on the same cancer cell samples.

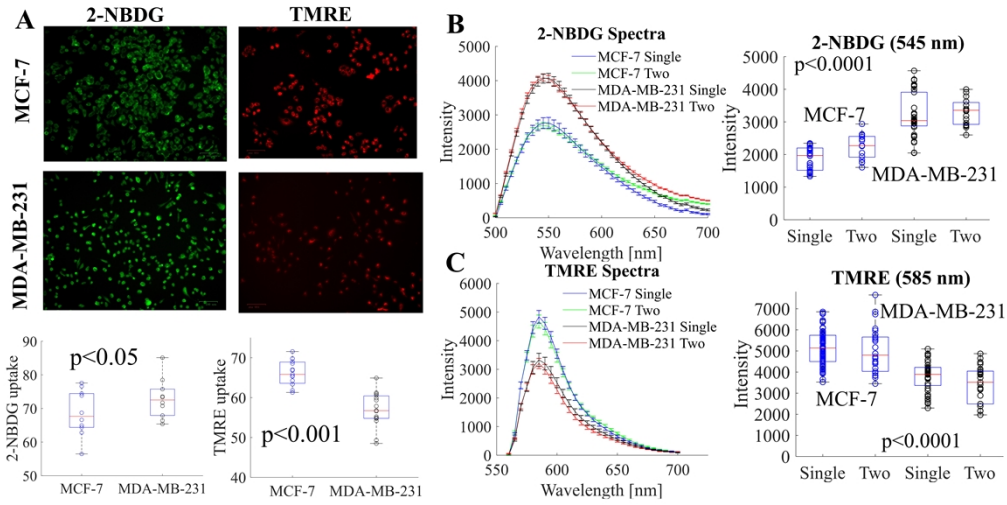

Fig. 4. **(A)** 2-NBDG and TMRE uptake in MCF-7 and MDA-MB-231 cell lines characterized by a fluorescence microscope. **(B)** Optical assay measured spectra of 2-NBDG in MCF-7 and MDA-MB-231 cell lines with single probe labeling approach and two-probe labeling approach. **(C)** Optical assay measured spectra of TMRE in MCF-7 and MDA-MB-231 cell lines with single probe labeling approach and two-probe labeling approach. The number of optical imaging for each set of 2-NBDG and TMRE uptake was around 20 (around 5 regions in each well for four different wells). The number of optical spectral measurements for each set of 2-NBDG and TMRE uptake was around 40 (around 10 regions in each well for four different wells). The cell confluence for the optical imaging and the optical assay measurements was maintained to be 80% for all measured cell wells.

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3.4. Optical spectroscopy captures metabolic changes of fresh tumor slices harvested from xenograft models

Figure 5 shows the optical assay measured glucose uptake (2-NBDG uptake) and MMP (TMRE uptake) of fresh SCC-61 tumor and normal tissue slices harvested from xenograft models. Figure 5(A) shows the representative fresh tissue samples harvested from xenograft models and their corresponding tumor slices (with a thickness of ~2 mm) in a cell culture plate. Figure 5(B-C) shows the comparison of fluorescence spectra between the single probe group (2-NBDG injection only or TMRE injection only) and the two-probes group (TMRE injection first and then 2-NBDG injection with a 15-minute delay) for both 2-NBDG and TMRE uptake measurements. Figure 5(B-C) shows that both the spectra shape and intensities were almost the same between the two experimental groups for both normal tissue slices and tumor slices. The statistical analysis on the 2-NBDG and TMRE uptake intensities for both normal tumor slices further demonstrated the two-probes approach was statistically identical compared to the single-probe approach. However, it is interesting to note that the shape of TMRE spectra but not 2-NBDG spectra measured on the tumor slices are slightly different compared to that measured on in vitro cells, which might be likely due to the distortions caused by the sample absorption or scattering given the sample thickness was around 2 mm. Nevertheless, Fig. 5(B-C) clearly shows that tumor slices have significantly increased 2-NBDG and TMRE uptake compared to that from normal tissue slices, which suggested that the tumor samples have upregulated glycolysis and mitochondrial metabolism compared to the normal tissues.

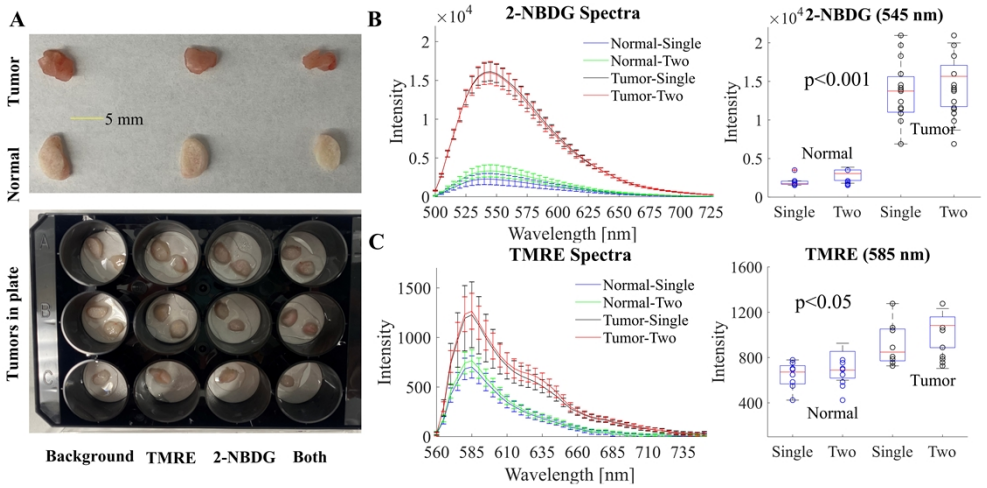

Fig. 5. **(A)** Representative tumor and normal samples harvested from xenograft models and their corresponding slices (with a thickness of 2 mm) in a cell culture plate for optical assay measurements. **(B)** Optical assay measured spectra of 2-NBDG in both normal and tumor slices with single-probe injection approach and two-probe injection approach. **(C)** Optical assay measured spectra of TMRE in both normal and tumor slices with single probe injection approach and two-probe injection approach. The number of optical assays for each set of 2-NBDG and TMRE uptake was around 9 (around 3 regions in the sample for three different samples). For the single-probe injection approach, either only TMRE or 2-NBDG stock was injected into mice through the tail vein and then waited for 1 hour for tumor harvest. For the two-probe injection approach, TMRE was injected first and then followed by 2-NBDG injection with a 15-minute delay based on our formerly published protocol and then waited for 1 hour for tumor harvest. The integration time for all spectral measurements was set to be 150 milliseconds.

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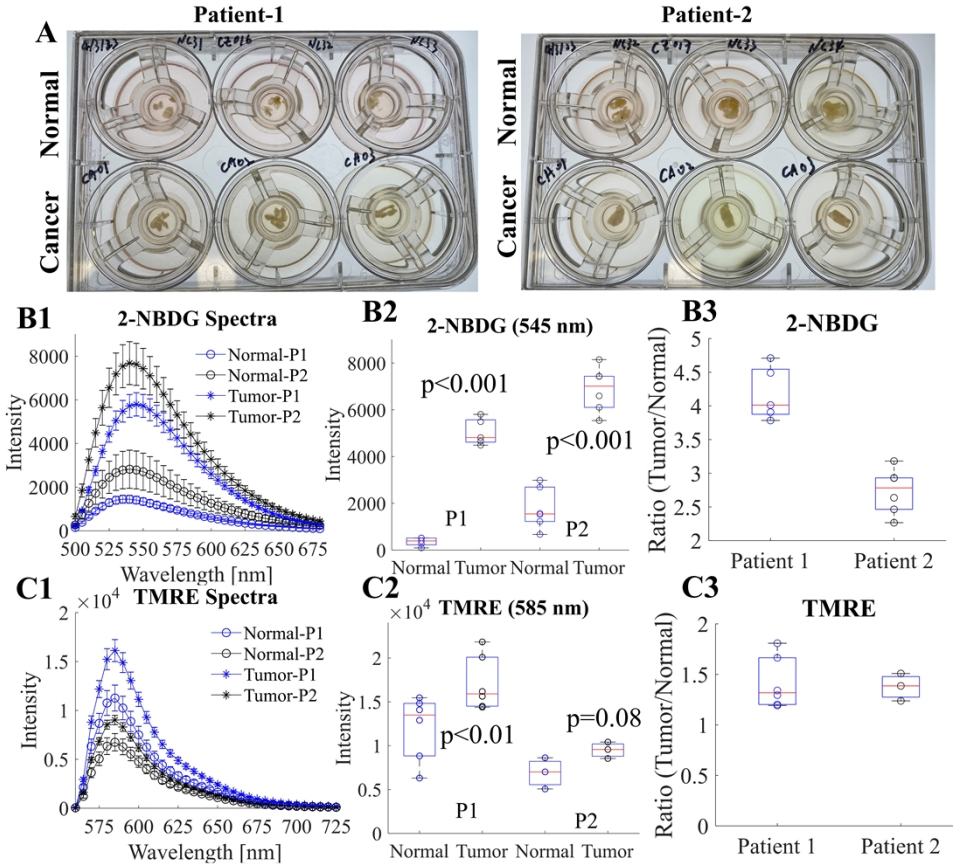

Fig. 6. **(A)** Representative tumor and normal samples harvested from two human breast cancer patients and their corresponding thin slices (with a thickness of 0.75 mm) in a cell culture plate for optical assay measurements. **(B)** Optical assay measured spectra of 2-NBDG in both normal and tumor slices for the two patient samples. **(C)** Optical assay measured spectra of TMRE in both normal and tumor slices for the two patient samples. The number of the optical assay for each set of 2-NBDG and TMRE uptake varied from 3 to 9 depending on the available measurable slices. The probe labeling on patient tumor slices was done using the protocol same as that used for *in vitro* cell studies. The integration time for all spectral measurements was set to be 150 milliseconds.

3.5. Optical assay captures increased metabolic activities of patient-derived tumor slices compared to normal tissue slices

Figure 6 shows the optical assay measured glucose uptake and MMP of fresh breast tumor and normal breast tissue thin slices harvested from two human breast cancer patients. Patient 1 (TCC7147) was diagnosed with ER + PR + HER2- IDC of the left breast without any treatment, while Patient 2 (TCC7145) was diagnosed with ER + with neoadjuvant treatments. Due to the limited number of patient samples and sample size, only single-probe labeling was used for the pilot optical assay measurements. Figure 6(A) shows the representative fresh tumor samples harvested from the two human breast cancer patients and their corresponding thin slices (thickness of 0.75 mm) in a cell culture plate. Figure 6(B1) shows that 2-NBDG uptake was significantly higher in tumor slices compared to normal tissue slices for both patient 1 and patient 2. Figure 6(C1) shows that TMRE uptake was significantly higher in tumor slices

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compared to normal tissue slices only for patient 1 but not for patient 2. Given the two patients have different cancer subtypes and have had different treatments, we did not intend to directly compare their metabolic profiles. However, we indeed captured that the two patients' samples have their own unique metabolic characteristics. It is noted that the shape of both TMRE and 2-NBDG spectra measured on the patient tumor and normal tissue slices are almost the same compared to that measured on *in vitro* cells, which suggested that the patient sample fluorescence signals had minimal distortions caused by sample absorption or scattering given the sample thickness was optically thin (~0.75 mm). To further illustrate the glycolytic and mitochondrial metabolic differences in tumors from the two patients, we normalized their tumors' 2-NBDG and TMRE uptakes by dividing their corresponding normal tissue slice probes uptakes. Figure 6(B3) shows patient 1 had significant tumor glucose uptake enhancement compared to patient 2, while Fig. 6(C3) shows that the two patients had comparable tumor TMRE uptake enhancement compared to their normal baseline. The normalized tumors' 2-NBDG uptakes suggest that the patient 1 tumor has enhanced glycolytic activities but not for patient 2, while the normalized tumors' TMRE uptakes suggest that the two patients did not have significantly enhanced mitochondrial activities.

4. Discussion

Increased glucose uptake and deregulated mitochondrial function have been explored for both tumor diagnosis and tumor therapeutic response predictions [2]. To investigate the role of tumor metabolism reprogramming in tumor growth and survival under therapeutic stresses, various tumor models are used for both cancer biology and tumor therapeutic investigations. The immortal cell model provides an easy-to-access platform to rapidly conduct cancer biology research [34]. However, it is challenging to translate potential treatments from cancer cell models to clinics due to the significant mismatch between the therapeutic responses of *in vitro* cells and patients [35]. The newly developed patient-derived organotypic tumor models [32] are suitable for rapid evaluation of therapeutic responses [36] as the organotypic tumor slices retain the original behavior and responsiveness of the *in vivo* tumors [8], making them the most clinically relevant model to examine tumor therapeutics responses [32]. Organotypic tumor slices are usually achieved within hours of surgical resection of the primary tumor by using a vibratome to slice the sample [32]. Dr. Gent's has reported a novel breast tumor slice culture system that can be used to assess drug response to primary breast tumors [32].

To provide direct evaluations on therapeutic responses of organotypic tumors before and after treatments and minimize the translational cost, it is critical to be able to conduct longitudinal non-destructive studies on the same population of the sample [9]. However, few techniques exist that can provide non-destructive metabolic measurements on patient organotypic tumor samples. The current existing metabolic tools that can be used for organotypic tumors samples characterization are Seahorse Assay [13,14] and metabolomics [15] but they are destructive tools. Recognizing the limitation of current technologies and the urgent need for non-destructive tools for metabolic characterization of patient-derived organotypic tumor samples, we reported a novel optical spectroscopic assay to enable non-destructive measurements of the key metabolic endpoints on *in vitro* cancer cells, fresh *ex vivo* tissue samples, and live patient-derived organotypic tumor slices. Preliminary studies showed that our portable optical assay may provide a cost-effective way to characterize the key metabolic parameters on *in vitro* cancer cells and organotypic fresh tumor slice models, which will significantly advance translational cancer research through the lens of tumor energetics.

The comparison study among our optical assay, a flow cytometry, and a benchtop microscope shows that the three techniques captured consistent metabolic changes among the MCF-7 (ER+) and MDA-MB-231 (triple-negative) cell lines. All three techniques captured that MDA-MB-231 cells had increased 2-NBDG uptake but decreased TMRE uptake compared to MCF-7 cells,

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which is supported by independent Seahorse Assay data [28] that showed the MDA-MB-231 cells have increased ECAR but decreased OCR compared to MCF-7 cell line. Flow cytometry provides single-cell level fluorescence measurements, but it is destructive, and the instrument is expensive. Microscope imaging can provide cellular resolution non-destructive metabolic imaging on single cells, while it is challenging to quantify the key metabolic parameters together on the same sample due to their limited spectral capability. The proposed spectroscopic assay enables simultaneous measurement of both glucose uptake and mitochondrial metabolism on the same sample but with the sacrifice of spatial information. The proposed spectroscopic assay offers an alternative approach for metabolism investigations on *in vitro* cells and organotypic tumor models, which may fill an important technology gap by providing an intermediate assessment capability between Seahorse Assay [13,14] and metabolomics [15]. In this proof-of-concept study, we primarily focused on the glucose uptake and MMP measurements due to the general interest in tumor glycolysis and mitochondrial function, and the glycolysis and mitochondrial function of the two breast cancer cell lines have been well characterized [19, 28]. It should be noted that our optical assay can be easily adapted to measure other metabolic probes such as fatty acid uptake using Bodipy [25] in our future work. Deconvolution algorithms [37] can be used to separate the Bodipy and 2-NBDG as the spectral characteristics of each probe are unique.

The fresh *ex vivo* tumor sample study using xenograft tumor models shows that the SCC-61 tumors have increased glucose uptake and mitochondrial activity compared to normal tissues. The comparison between the single-probe and the two-probe approach showed that it is feasible to measure both glucose uptake and mitochondrial function on the same tumor sample using the spectroscopic approach. However, it is noted that the measured fluorescence signal may be distorted by tissue absorption or scattering if the tumor slice is thick. Therefore, thick tissue samples should be avoided for future use of the spectroscopic assay. Proper fluorescence signal correction [38] may help address such distortion if a thick sample investigation is involved. For the first time, we utilized human patient-derived organotypic breast tumor slices to evaluate our spectroscopic assay for metabolic characterizations. Though only two patient samples were investigated in this pilot study, our assay can capture their unique metabolic differences between the tumor sample and normal tissues. The pilot studies showed that the two patients' tumors have enhanced glycolytic activities, while their tumors did not have significantly enhanced mitochondrial activities. Patient 1 was diagnosed with ER + PR + HER2- IDC without any treatment which may explain the significantly enhanced glycolytic activities, while patient 2 was diagnosed with ER + but has received a neoadjuvant treatment which may explain the relatively lower enhancement of glycolytic activities compared to patient 1. It is interesting to highlight that the patient 2 who has received a neoadjuvant treatment still had enhanced glycolysis compared to their normal baseline, which could be because the treated tumors have immune cell recruitment, leading to potentially higher 2-NBDG uptake [39]. The normalized tumors' TMRE uptake suggests that the two patients' tumor samples only had marginal enhancement of mitochondrial activities (~1.5 times compared to normal tissues) which may inform that the two patients' tumors rely more on glycolysis rather than mitochondrial metabolism. We optimized the patient tumor slice thickness to be 0.75 mm in the slice culture system, which enabled us to keep the sample alive for as long as four weeks. We noticed that the measured fluorescence signal has not been distorted by tissue absorption or scattering if the tumor slice is optically thin, which will provide useful guidance for future similar investigations using thin organotypic tumor slices.

In this study, we kept the tumor slices always larger than the optical sampling area (1 mm in diameter) to avoid any potential experimental errors. However, the sizes or shapes of tumor slices should be considered for future experiments as the fluorescence spectra could be distorted if the tissue slice size is smaller than the sampling area. Given that, we would recommend to keep the tumor slices always larger than the optical sampling area. A fiber probe with a smaller sampling area should be used for slices with a small size. Due to the limited number of patient samples

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and sample size in this proof-of-concept pilot clinical sample study, only single-probe labeling was used for the pilot optical assay measurements. However, it should be noted that it is feasible to quantify the key metabolic parameters simultaneously on the same clinical tumor slices, which will be explored in our future study plan. The combination of multiple metabolic parameters (2-NBDG and TMRE in our case) measured on the same sample could be explored as a new way for future data analysis as reported by others before [9].

In this study, a single-channel optical assay was reported to demonstrate the proof-of-concept, while a multi-channel optical assay can be easily upgraded to increase the throughput for rapid metabolic characterization on organotypic tumor slices in our future study plan. We demonstrated the single-channel optical assay on *in vitro* breast cancer cells, fresh tumor slices harvested from animals, and live thin tumor slices harvested from human patients for both glucose uptake and mitochondrial metabolism characterizations. These demonstrations showed that the reported optical metabolic assay could be a useful non-destructive tool for metabolic investigations on *in vitro, ex vivo*, and organotypic tumor models. Moreover, our optical assay is highly portable and low-cost thanks to the new compact optoelectronic components, thereby potentially provide point-of-care metabolic measurements on tumor models with high user access in the future, which will significantly advance transitional cancer applications.

5. Conclusion

This work reports a novel portable cost-effective optical spectroscopic assay for non-destructive metabolic characterizations on various tumor models. We demonstrated the technique for measurement of key metabolic parameters on *in vitro* cancer cells, *ex vivo* tumor samples, and organotypic fresh tumor slice models. The technique will potentially enable longitudinal therapeutic studies on the same organotypic tumor samples through the lens of tumor energetics, which will significantly advance translational cancer research.

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Data availability. Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

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